APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1989, p. 1295–1297
0099-2240/89/051295-03$02.00/0

Vol. 55, No. 5

Excretion of Amino Acids by 1,2,4-Triazole-3-Alanine-Resistant Mutants of *Methanococcus voltae*

KAREN A. SMENT AND JORDAN KONISKY*
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 28 November 1988/Accepted 8 February 1989

In contrast to wild-type cells, it was found that triazole-alanine-resistant mutants of *Methanococcus voltae* excreted histidine, proline, phenylalanine, and tyrosine in various combinations. These results suggest that a form of general amino acid biosynthetic control may operate in this methanogen. We also show that wild-type *M. voltae* excretes methionine.

In order to isolate *Methanococcus voltae* mutants altered in the regulation of histidine biosynthesis (12) and to provide genetic markers for genetic studies, we selected mutants resistant to 1,2,4-triazole-3-alanine (TRA). This selective agent has been used to isolate regulatory mutants of *Salmonella typhimurium* (10, 11).

A culture of *M. voltae* PS (DSM 1537) was grown anaerobically under a pressurized H_2-CO_2 (80:20) atmosphere in defined medium (4, 13) to mid-log phase, harvested anaerobically by centrifugation, and suspended in one-half of its original volume of 0.4 M NaCl. In an anaerobic chamber (Coy Manufacturing), approximately 8×10^6 cells (0.1 ml) were mixed with 3 ml of 0.7% agar in defined medium containing 19 amino acids (no histidine), 0.4 mM adenine, and 0.8 mM 3-amino-1,2,4-triazole (aminotriazole), and the suspension was poured on a defined agar medium plate containing the same additions. Aminotriazole, which inhibits the histidine biosynthetic enzyme, imidazoleglycerol phosphate dehydratase, was added to reduce histidine production sufficiently to render the cells sensitive to TRA (1, 6, 9, 10). A 6-mm-diameter sterile filter disk containing either 0.8, 1.6, or 3.2 μmol of TRA in 40 μl of sterile water was placed in the center of each plate, and the plates were incubated anaerobically for 1 week at 37°C in brass-stainless steel canisters under a pressurized H_2-CO_2 (80:20) atmosphere containing H_2S (final concentration, 0.2% [vol/vol]). Double mutants were isolated from cultures of the TRA-resistant mutant KS8-1 (see below) by selecting for mutants resistant to higher levels of TRA by repeating this procedure but using filter disks impregnated with either 6 or 12 μmol of TRA.

Seven randomly selected TRA-resistant mutants growing within the zone of inhibition and wild-type *M. voltae* were tested for their levels of resistance to TRA. Wild-type cells did not grow within 24 h in the presence of 1 μM TRA. In contrast, mutants KS8-1, KS51-4, and KS24-2 were resistant to 100, 200, and 600 μM TRA, respectively. The remaining mutants grew in the presence of TRA at a concentration of at least 800 μM. The spontaneous double mutants derived from KS8-1 (KS815-5, KS817-5, and KS822-5) were resistant to TRA at a concentration of at least 800 μM.

Since we reasoned that TRA resistance might have resulted from mutations which led to enhanced production of histidine, we determined whether each mutant excreted histidine to the extent that it cross-fed an *Escherichia coli* histidine auxotroph. Both the *E. coli* auxotroph and the methanogen were streaked across a defined agar medium plate containing 0.2% glucose (Fig. 1). While the presence of wild-type *M. voltae* did not enhance growth of the auxotrophic strain, the auxotrophic strain was rescued by the TRA-resistant mutant, presumably by the excretion of histidine. In control experiments, it was shown that the addition of histidine alone was sufficient to allow growth of the auxotroph in the absence of added methanogenic cells.

The mutants were also examined for their ability to rescue arginine, tyrosine, proline, methionine, threonine, phenylalanine, tryptophan, or purine auxotrophs. A typical experiment is shown in Fig. 1B, where it can be seen that while wild-type *M. voltae* did not rescue an *E. coli* proline auxotroph, the mutant KS817-5 excreted sufficient proline to support growth of the *E. coli* mutant. Neither the TRA-resistant mutants nor the wild-type strain rescued arginine, threonine, tryptophan, or purine auxotrophs. However, some mutants excreted various combinations of proline, phenylalanine, and tyrosine (Table 1).

Both wild-type *M. voltae* and the mutants rescued an *E. coli metB1* strain, suggesting that *M. voltae* excretes L-methioine or perhaps the biosynthetic intermediates, cystathionine or homocysteine. Our finding that the spent medium of an *M. voltae* culture rescued an *E. coli metE* auxotroph which is defective in the terminal step of methionine biosynthesis (Table 2) indicates that *M. voltae* was not supplying histidine pathway intermediates.

To rule out the possibility that rescue of the *metE* auxotroph resulted from the excretion of vitamin B_{12} and the subsequent production of methionine in the *E. coli* auxotroph via the vitamin B_{12}-dependent *metH*-encoded methylase pathway (3), we examined a *metE* strain which is unable to take up vitamin B_{12} owing to defects in the *btuB* and *btuC* genes (5, 8). While addition of vitamin B_{12} (50 μg/liter) did not overcome this auxotroph's methionine requirement, it was rescued by the addition of either methionine (80 mg/liter) or *M. voltae* spent medium (Table 2).

While it is highly unlikely that *M. voltae* excretes methionine fortuitously, we have little notion of its significance. However, it is interesting that it has been suggested that methionine is an important intermediate in the formation of organic thiols in marine sediments (7). Whether excreted

* Corresponding author.

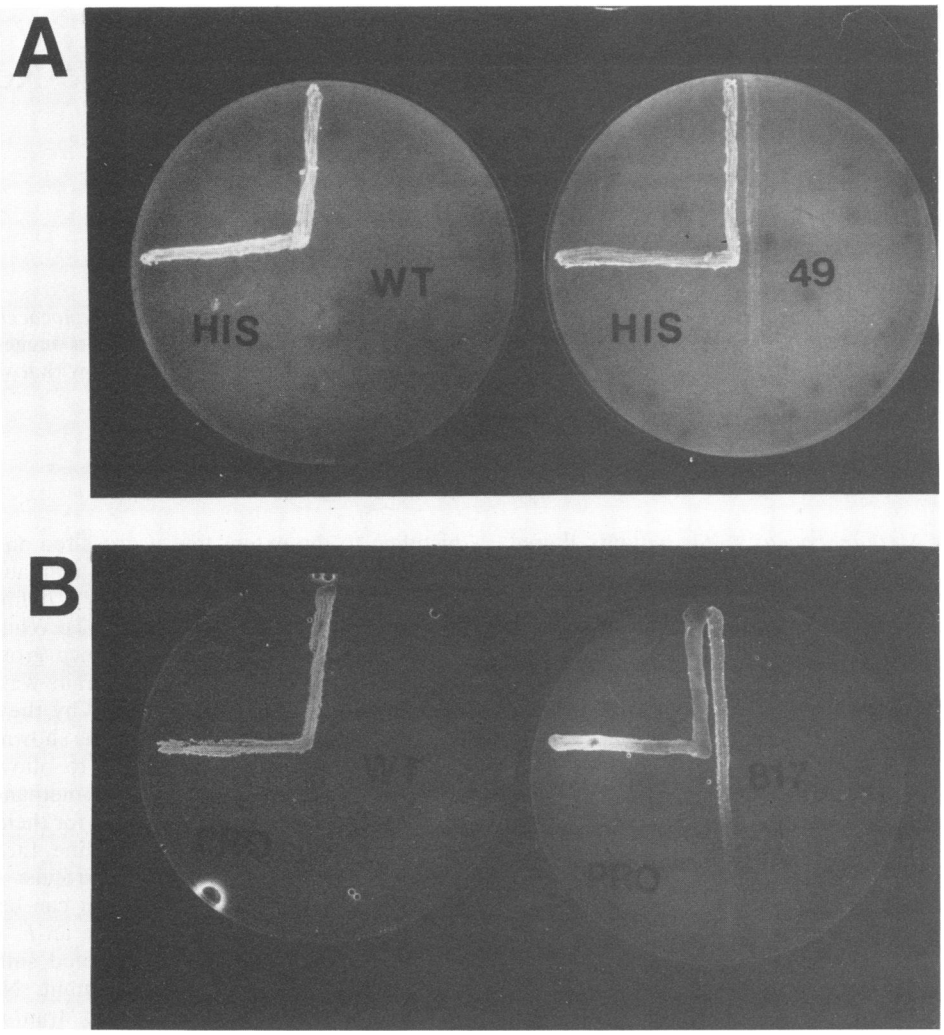

FIG. 1. Cross-feeding plate assay. (A) The *E. coli* histidine auxotroph JK677 was streaked in a vertical line on both plates. Wild-type *M. voltae* (left plate) and KS49-4 (right plate) were streaked in an L shape next to the auxotroph. (B) The *E. coli* proline auxotroph CGSC 4513 was streaked next to the wild-type *M. voltae* and KS817-5.

methionine is recycled via the formation of such organic thiols must await further study.

The fact that spontaneous mutations can lead to the overproduction of several unrelated amino acids suggests that *M. voltae* possesses a general control system for the regulation of amino acid biosynthesis similar to that found in *Saccharomyces cerevisiae* (2). However, further work is clearly needed to verify such a possibility. If confirmed, it

TABLE 1. Cross-feeding by TRA-resistant mutants[a]

Strain	Cross-feeding by auxotrophs[b]				
	His	Met	Phe	Pro	Tyr
Wild-type	−	+	−	−	−
KS4-1	+	+	−	+	−
S8-1	−	+	−	+	−
S12-1	+	+	−	−	+
KS24-2	+	+	−	+	+
KS49-4	+	+	−	+	+
KS51-4	+	+	+	+	+
KS63-4	+	+	+	+	+
KS815-5	+	+	+	+	+
KS817-5	+	+	−	+	+
KS822-5	+	+	−	−	−

[a] Auxotroph tested: *argH his metB1 pheA proB purF thrB trp*::Tn*10 tyrA*.
[b] Symbols: +, cross-feeding; −, no cross-feeding.

TABLE 2. Rescue of *E. coli* methionine auxotrophy by *M. voltae*

Auxotroph	Medium[a]	Addition(s)	Rescue[b]
metE	Fresh	−Met	−
	Fresh	+Met	+
	Test	−Met	+
metE btuB btuC	Fresh	−Met + B_{12}	−
	Fresh	+Met + B_{12}	+
	Fresh	−Met	−
	Fresh	+Met	+
	Test	−Met	+

[a] Positive and negative controls were tested in fresh medium in which no methanogen had grown. The medium in which *M. voltae* had grown served as the test medium.
[b] Symbols: −, no growth of the auxotroph after 18 h of incubation at 37°C; +, the auxotroph went through at least seven doublings after incubation for 18 h.

would provide yet another example in which a property of an archaebacterium is more eukaryotic than eubacterial (14).

This work was supported by Department of Energy research grant DEFG 0284 ER 132 and National Science Foundation grant BB58714603.

LITERATURE CITED

1. **Hilton, J. L., P. C. Kearney, and B. N. Ames.** 1965. Mode of action of the herbicide, 3-amino-1,2,4-triazole (Amitrole): inhibition of an enzyme of histidine biosynthesis. Arch. Biochem. Biophys. **112**:544–547.
2. **Hinnebusch, A. G.** 1987. The general control of amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. Crit. Rev. Biochem. **21**:277–317.
3. **Jeter, R. M., B. M. Olivera, and J. R. Roth.** 1984. *Salmonella typhimurium* synthesizes cobalamin (vitamin B_{12}) *de novo* under anaerobic growth conditions. J. Bacteriol. **159**:206–213.
4. **Jones, W. J., W. B. Whitman, R. D. Fields, and R. S. Wolfe.** 1983. Growth and plating efficiency of methanococci on agar media. J. Bacteriol. **46**:220–226.
5. **Kadner, R. J., and B. L. Liggins.** 1973. Transport of vitamin B_{12} in *Escherichia coli*: genetic studies. J. Bacteriol. **115**:514–521.
6. **Levin, A. P., and P. E. Hartman.** 1963. Action of a histidine analogue, 1,2,4-triazole-3-alanine, in *Salmonella typhimurium*. J. Bacteriol. **86**:820–828.
7. **Mopper, K., and B. F. Taylor.** 1986. Biogeochemical cycling of sulfur: thiols in coastal marine sediments, p. 324–339. *In* M. Sohn (ed.), Organic marine geochemistry. American Chemical Society Symposium Series. American Chemical Society, Washington, D.C.
8. **Reynolds, P. R., G. P. Mottur, and C. Bradbeer.** 1980. Transport of vitamin B_{12} in *Escherichia coli*: some observations on the roles of the gene products of *btuC* and *tonB*. J. Biol. Chem. **255**:4313–4319.
9. **Roth, J. R., D. N. Anton, and P. E. Hartman.** 1966. Histidine regulatory mutants in *Salmonella typhimurium*. I. Isolation and general properties. J. Mol. Biol. **22**:305–323.
10. **Rudd, K. E., B. R. Bochner, M. Cashel, and J. R. Roth.** 1985. Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on *his* operon expression. J. Bacteriol. **163**:534–542.
11. **Silbert, D. F., G. R. Fink, and B. N. Ames.** 1966. Histidine regulatory mutants in *Salmonella typhimurium*. III. A class of regulatory mutants deficient in tRNA for histidine. J. Mol. Biol. **22**:335–347.
12. **Sment, K. A., and J. Konisky.** 1986. Regulated expression of the *Methanococcus voltae hisA* gene. Syst. Appl. Microbiol. **7**: 90–94.
13. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
14. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–272.